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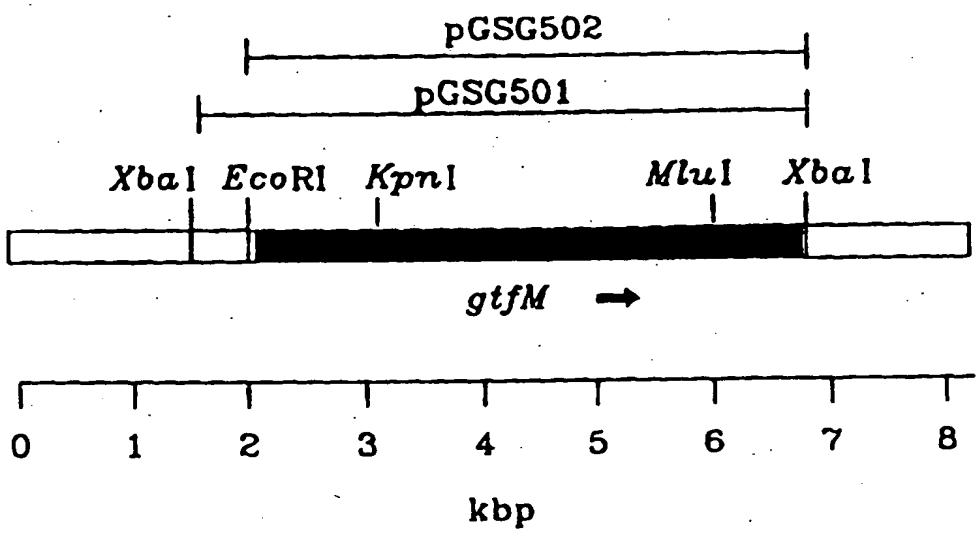

(57) Abstract

The present invention relates to plants genetically modified to increase the level of stored carbohydrates in the plant, particularly during periods of high sink activity and low source activity through production of a glycosyl-transferase which catalyses the formation of soluble glucans. The invention also relates to the genetic constructs used to produce the engineered plants and the method of producing the engineered plants.

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"Genetic Manipulation of Plants to Increase Stored Carbohydrates"

TECHNICAL FIELD

The present invention relates to plants genetically modified to increase the level of stored carbohydrates in the plant, particularly during periods of high sink activity and low source activity. The invention also relates to the genetic constructs used to produce the engineered plants and the method of producing the engineered plants.

BACKGROUND ART

The soluble storage carbohydrate found in plants, including sucrose, glucans, starch and fructans, are an important source of feed for animals, particularly grazing ruminants. These carbohydrates are stored non-structurally which makes them readily available for digestion by animals and therefore an important source of digestible energy.

During periods of high sink activity and low source activity, such as during a drought, the level of stored carbohydrates falls as the non-structural storage carbohydrates are mobilised for use in seed filling. The result of this mobilisation, particularly in relation to pasture grasses, is a significant loss of feed value to grazing ruminants due to the reduction in the levels of the stored carbohydrates. This reduction is caused by the enzymatic degradation of the stored carbohydrates. This enzymatic degradation is assisted by the fact that the stored carbohydrates generally have a low degree of polymerization. For example, as noted by Radojevic et al 1994, during the period from late spring to early autumn in southern Australia, the declining feed quality of the grasses causes a corresponding reduction in the lactation by dairy herds and necessitates the use of supplementary feeds. This decline in digestibility is associated with a decline in the level of soluble carbohydrates. Perennial rye grass lines which accumulate high concentrations of soluble carbohydrates from late spring

to early autumn do not suffer as large a decline in digestibility (Radojevic et al 1994). The result of this increased digestibility is a corresponding increase in milk production by dairy herds.

In addition to this, there are many pasture plants, such as white clover which do not possess any significant levels of stored carbohydrate.

There has, therefore, been a desire to develop methods for preventing the degradation of the stored carbohydrates during plant senescence and to increase the level of stored carbohydrates in pasture plants with low levels.

Glucosyltransferases of *Streptococcus salivarius*

It is known that many strains of *Streptococcus salivarius* and *Streptococcus mutans*, produce extracellular α-D-glucosyltransferase (Gtfs), an enzyme which catalyses the formation of glucan from sucrose. These Gtfs are also found in many other species of oral streptococci.

The Gtfs utilise the high free energy of the glycosidic bond of sucrose to synthesise glucans (Jacques NA, Giffard PM, 1991). Gtfs produce either soluble or insoluble products by transferring a glucose residue from sucrose to a growing glucan chain.

Gtfs which produce an insoluble product are generally considered to be primer-dependent (Walker GJ, Jacques NA, 1987). These primer-dependent Gtfs require a dextran (α-(1→6)-linked glucan) as a receptor for polymerisation to proceed at an appreciable rate. In contrast, Gtfs that produce soluble products may be either primer-dependent or primer-independent.

The genetic sequences for 10 gtf genes from a number of *Streptococcus* species have been ascertained (Gilmore KS, Russell RRB, Ferretti JJ). All the Gtfs coded by these genes possess highly conserved putative signal sequences that lead to the secretion of these enzymes.

The remainder of each protein is arbitrarily divided into two domains - the N-terminal two-thirds "catalytic domain" and the C-terminal one-third "glucan-binding domain".

S. salivarius ATCC 25975 has been shown to possess at least four different gtf genes (Giffard et al (1991); Giffard et al (1993)). Each of these genes codes for a highly hydrophilic monomeric glucosyltransferase that possesses unique enzymic properties. These Gtfs synthesize structurally different glucans from sucrose. For example, the genes coding for GtfJ and GtfL produce enzymes which synthesize insoluble glucans. GtfJ is a primer-dependent enzyme producing essentially a linear $\alpha(1\rightarrow3)$-glucan while GtfL is a primer-independent enzyme that synthesizes a glucan containing 50% α-(1→3)- and 50% α-(1→6)-linked glucosyl residues. In contrast, the gtfK and gtfM genes code for enzymes which produce a soluble glucan which possess α-(1→6)-linked glucosyl residues. GtfK is primer stimulated while GtfM is primer independent.

DESCRIPTION OF THE INVENTION

Up until now, a gtf gene in *S. salivarius* or any other *Streptococcus* species which produces a glucosyltransfererase that synthesises a glucan which is both soluble and primer independent has not been described.

The significance of a glucosyltransferase produced by *S. salivarius*, or any other streptococci, which is both primer independent and which synthesises a soluble glucan product is twofold. First, the primer independence of the Gtf means that the enzyme should be functional when expressed in plants while the glucan that is formed from sucrose in the plant should be readily stored without detriment to the plant, due to its solubility.

An important characteristic of soluble glucans produced by Gtf synthesis is that they are poorly degraded by plant enzymes and are readily digested by the diverse microflora present in the rumen of grazing livestock.

The inventors of the present invention have isolated and characterised a novel gtf (GtfM) gene in *S. salivarius* which codes for a primer independent Gtf which produces a glucan which is soluble, resistant to degradation by plant enzymes and readily digested by microflora present in the rumen of grazing livestock.

According to a first aspect of the present invention there is provided a plant containing bacterial DNA which codes for a glucosyltransferase which catalyses the formation of glucans from sucrose.

Preferably, the plant contains bacterial DNA which codes for a glucosyltransferase which is primer independent.

More preferably, the plant contains DNA which codes for a glucosyltransferase which catalyses the formation of soluble glucans.

More preferably, the bacterial DNA is obtained from *Streptococcus salivarius*.

According to a second aspect of the present invention there is provided a DNA comprising a sequence according to SEQ ID NO: 1.

According to a third aspect of the present invention there is provided a DNA sequence which is a variant of a DNA having a sequence according to SEQ ID NO: 1. In this respect a "variant" is a polynucleotide which corresponds to or comprises a portion of the DNA of the invention, or is "homologous" to the DNA of the invention. For the purposes of this description, "homology" between two polynucleotide sequences connotes a likeness short of identity, indicative of a derivation of the first sequence from the second. In particular, a polynucleotide is "homologous" to the DNA of the

invention if there is greater than 70% identity in the DNA sequence.

The polynucleotides of the present invention exclude those polynucleotides in the environment in which they occur in nature. They include the polynucleotides in a form in which they are substantially free of other *Streptococcus salivarius* polynucleotide sequences, such as sequences in isolated form, including those in substantially purified form.

According to a fourth aspect of the present invention there is provided a protein comprising the amino acid sequence according to SEQ ID NO: 2.

According to a fifth aspect of the invention there is provided a polypeptide comprising an amino-acid sequence which is a variant of SEQ ID NO:2. A variant is a polypeptide which corresponds to or comprises a portion of the polypeptide of the invention, or is "homologous" to the peptide of the invention. For the purposes of this description, "homology" between two peptide sequences connotes a likeness short of identity, indicative of a derivation of the first sequence from the second. In particular, a polypeptide is "homologous" to the peptide of the invention if there is greater than 70% identity in the amino acid sequence.

These homologous polypeptides can be produced by conventional site-directed mutagenesis of the corresponding DNA or by chemical synthesis, and fall within the scope of the invention, particularly where they retain the biological activity of a glucosyltransferase.

The proteins and polypeptides of the invention exclude those proteins and polypeptides in the environment in which they occur in nature. They include the proteins and polypeptides in a form in which they are substantially free of other *Streptococcus salivarius* polypeptide sequences, such as sequences in isolated form, including those in substantially purified form.

According to a sixth aspect of the present invention there is provided the microorganism *E. coli* containing plasmid pGSG501.

According to a seventh aspect of the present invention there is provided the microorganism *E. coli* containing plasmid pGSG502.

According to a eighth aspect of the present invention there is provided a plant containing DNA comprising a sequence according to SEQ ID NO: 1.

According to an ninth aspect of the present invention there is provided a plant containing DNA which is a variant of DNA having a sequence according to SEQ ID NO: 1.

According to a tenth aspect of the present invention there is provided a plant expressing a protein comprising an amino acid sequence according to SEQ ID NO: 2 or a variant thereof.

DNA and variants thereof of the invention can be incorporated into a variety of plant types. These include plants, such as grasses, used as fodder for livestock. They also include cereal crops or other starchy food product types, (to provide grain or other food with increased fibre); and horticultural crops, such as tomatoes and fruits, to provide fruits with increased solids.

In addition plants expressing the DNA and variants thereof, of the invention may also produce dextran which can in turn be used:

1) as a binder for use in processed foods (e.g. so called 'health bars');
2) in pharmaceutical preparations again as a binder; and
3) in medical preparations to increase antigenic activity.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a restriction map of the inserts from pGSG501 and pGSG502.

BEST METHOD OF PERFORMING THE INVENTION

The invention is further described with reference to the accompanying Example which is no way limiting on the scope of the present invention.

Example 1

The general strategy adopted to isolate a gene from *S. salivarius* encoding a Gtf which produces a primer independent and soluble glucan is as follows:

A λ gene bank containing *S. salivarius* DNA was prepared. Positive clones were detected by using an *E. coli* strain grown on agar containing sucrose. *E. coli* which contained gtf DNA from *S. salivarius* could convert the sucrose in the medium into a polymer which resulted in opaque colonies. These opaque colonies were then picked and the *S. salivarius* DNA excised and subjected to restriction mapping to ascertain whether the DNA was from a previously described *S. salivarius* gtf gene, or whether the DNA was novel. Three clones containing novel DNA were located. These were subjected to a radioactive assay to determine whether the DNA encoded for a primer independent or primer dependent Gtf. One clone-λC-13 was found to contain a novel gtf gene which coded for a primer independent Gtf. The DNA from this clone was then isolated and sequenced.

The particular details of this methodology are now described below.

Bacterial strains and growth conditions.

Escherichia coli LE392 and NM522 and *S. salivarius* ATCC 25975 were used. *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C, supplemented with ampicillin (100 $\mu g\ ml^{-1}$), isopropylthiogalactoside (IPTG) (1mM), or 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (100

$\mu g\ ml^{-1}$) as appropriate. Cultures of *S. salivarius* were grown at 37°C in semi-defined medium (SDM) containing 25 mM glucose supplemented with 0.005μl Tween 80 ml^{-1} where appropriate.

Bacteriophage and phagemids. All genetic constructs, excluding sequencing subclones, are listed in Table 1. Bacteriophage-λ derivatives were grown either as 20 ml or 1 L-liquid lysates using *E. coli* strain LE392 as the host and DNA purified according to the method of Silhavy et al (1984). Plasmids were propagated in *E. coli* strains as described previously (Giffard et al, 1991).

Screening of Gene Bank. A bacteriophage-λ gene bank of *S. salivarius* ATCC 25975 (Pitty et al, 1989) was screened by detecting plaques on a lawn of *E. coli* LE392 grown at 37°C on minimal agar medium containing 0.2% glucose and 50 $\mu g\ ml^{-1}$ methionine as well as 1% (wt/vol) sucrose with or without 0.02% (wt/vol) dextran T-10. Potential *Gtf* clones were detected by their opacity including λ C-13 containing the gtf M gene.

Twenty recombinant plaques were picked from minimal media plates containing sucrose and the EcoR1 restriction patterns of these recombinants were analysed. Of these recombinants, only λC-13 exhibited a unique EcoR1 restriction pattern and Gtf activity. A restriction map of λC-13 was constructed using double restriction digests. The Gtf gene encoded by λC-13 (GtfM) was located on an 8.3 kbp insert (see figure 1). The 5.3 kbp XbaI fragment from λC-13 was subcloned into pIBI31 (pGSG501; see Table 1) and was positive for Gtf activity as was the 4.8 kbp XbaI/EcoR1 from λC-13 subcloned into pIBI31 (pGSG502; see Table 1).

Table 1. Bacterial Strains, Phages and Phagemids

Bacteria, Phage or Phagemid	Description	Source or reference
Bacterium:		
Streptococcus salivarius ATCC 25975		ATCC (Hamilton, 1967).
Escherichia coli LE392	F- e14-(mcrA-) *hsdR514* ($r_K^- m_K^+$) *supE44 supF58 lacY1* or Δ(*lacIZY*)*6 galK2 galT22 metB1 trpR55*	Murray *et al.*, 1977
Escherichia coli NM522	F'*lacI*^{q}Δ(*lacZ*) *M15 proA*$^+$*B*$^+$/*supE thi* Δ(*lac-proAB*) Δ(*hsdMS-mcrB*)*5* ($r_K^- m_K^-$ McrBC)	Gough and Murray, 1983
Bacteriophage:		
λL47.1		Loenen and Brammar, 1980
λA-8	λL47.1 with GtfJ encoding 8.5kbp *Sau*3A partial fragment of *S. salivarius* ATCC 25975	Pitty *et al.*, 1989
λA-33	λL47.1 with GtfK encoding 9.6kbp *Sau*3A partial fragment of *S. salivarius* ATCC 25975	Pitty *et al.*, 1989
λC-13	λL47.1 with 8.3kbp GtfM encoding *Sau*3A partial fragment of *S. salivarius* ATCC 25975	This study
λD-10	λL47.1 with 11kbp GtfL encoding *Sau*3A partial fragment of *S. salivarius* ATCC 25975	This study
λD-40	λL47.1 with *Sau*3A partial fragment of *S. salivarius* ATCC 25975 isolated from sucrose-containing medium	This study
Phagemid:		
pIBI30	Ap^r, f1 origin replication, β-galactosidase, T3 and T7 polymerase promoters	IBI Corporation
pIBI31	Ap^r, f1 origin replication, β-galactosidase, T3 and T7 polymerase promoters	IBI Corporation
pGSG101 (pGS101)	pIBI30 with GtfJ encoding 6.8kbp *Sac*I/*Bam*HI fragment of λA-8	Giffard *et al.*, 1991
pGSG201 (pGS201)	pIBI30 with GtfK encoding 7.3kbp *Bgl*II/*Bam*HI fragment of λA-33	Giffard *et al.*, 1991
pGSG401	pIBI30 with GtfL encoding 6.2kbp *Bam*HI/*Xba*I fragment of λD-10	This study
pGSG402	pIBI31 with 6.2kbp *Bam*HI/*Xba*I fragment of λD-10	This study
pGSG403	pIBI30 with 4.8kbp *Eco*RI fragment of λD-10	This study
pGSG404	pIBI30 with 4.1kbp *Eco*RI fragment of λD-10	This study
pGSG501	pIBI31 with GtfM encoding 5.3kbp *Xba*I fragment of λC-13	This study
pGSG502	pIBI31 with GtfM encoding 4.8kbp *Eco*RI/*Xba*I fragment of λC-13	This study
pGSG503	pIBI31 with 3.7kbp *Kpn*I/*Xba*I fragment of λC-13	This study

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Detection of Gtf activity. Gtf activity was routinely detected using a qualitative microtitre reducing sugar test for liberated fructose, outlined in Jacques N.A. (1983). Gtf activity encoded by phagemids was released from *E. coli* cells by permeabalizing 1 ml of a stationary phase culture. This was achieved by vortexing the cells in the presence of 50 µl 0.1% (wt/vol) SDS and 100 µl chloroform for 20 seconds. Quantification of Gtf activity utilized [U-glucosyl-^{14}C]-labelled sucrose. One unit of enzyme activity was defined as the amount of Gtf that catalyzed the incorporation of 1µmol of the glucose moiety of sucrose in 75% (vol/vol) ethanol-insoluble polysaccharide per min.

The assay mix used for the quantification of Gtf activity was scaled up to 8 ml and incubated with 3.2ml of bacteriophage λ lysates at 37°C for 2h. After the 2h incubation, the assay mix was boiled for a further 1h to inactivate the enzyme and the amount of glucan formed (cpm) determined by assaying duplicate 500µl aliquots. After cooling to 37°C, *C. gracile* endo-(1→6)-α-D-glucanase was added to a final concentration of 500mU/ml and the solution incubated at 37°C. Duplicate aliquots (500µl) were removed and assayed for total remaining glucan at varying time intervals over a 5h period. Any reduction in glucan (cpm) during this period was attributed to hydrolysis by the endo-(1→6)-α-D-glucanase.

DNA sequence analysis. DNA sequence determination was carried out on CsCl purified double-stranded DNA using the Pharmacia T7 sequencing kit according to the manufacturer's instructions. Custom-made oligonucleotide primers (17mers) were used and all sequencing was confirmed in both directions. DNA sequences were assembled and open reading frames (orfs) detected using the IBI-Pustell sequence analysis software version 2.03.

Southern Hybridizations. Chromosomal DNA from *S. salivarius* ATCC 25975 was extracted and purified as

previously described (Giffard et al, 1991). Southern hybridizations were done essentially as outlined by Silhavy et al (1984) and in accordance with standard techniques such as those described in Maniatis et al (1989).

Incorporation into plants. Incorporation of gtfM gene into plants is obtained by standard transgenic techniques. The gtfM gene is obtained from λC-13 or pGSG501 by PCR. Various constructs are made using PCR primers that either do or do not contain a coding region that adds a vacuolar targeting sequence to the N- or C-terminus of the GtfM protein. These PCR constructs are cloned into a pUC18 based vector containing a Cauliflower Mosaic Virus (CaMV) 35S promoter. By this means the streptococcal promoter is replaced by a plant promoter.

Other methods of incorporating foreign DNA into plants are taught in Australian Patent Application No. 46881/89 by Ciba Geigy Ag. They include the use of Agrobacterium tumefaciens and the leaf disc transformation method and the use of Tobacco Mosaic Virus (TMV).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: Genetic Manipulation of Plants to Increase Stored Carbohydrates

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4853 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Streptococcus salivarius

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CAGAGATTTA TGAAAAGAAG ATGATTTTTT CCTATTTGTA ATTTGTCTGA ATATATCATA 60

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CCTAACGGTG ACGGCTTGCA GCAACTGAGT GAGGATGGGA CTGCCAGTCT AGTGACGACA 300

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GTTAAGACAC CAAGTCAGTA CACCAACCGT GTTATTGCTC AAAATGCCAA ACTCTTCAAA 3000

GAGTGGGGAA TCACTTCCTT TGAATTTGCG CCACAGTATG TGTCTAGCCA AGACGGCACT 3060

TTCTTGGATT CTATCATTGA AAATGGCTAC GCCTTCGAGG ACCGCTACGA TATTGCCATG 3120

AGCAAGAACA ACAAATACGG TTCACTCAAA GACCTCATGG ATGCCCTTCG TGCCCTTCAC 3180

GCAGAAGGTA TCTCAGCCAT TGCTGACTGG GTTCCAGATC AAATTTACAA TCTCCCTGGA 3240

AAAGAAGTGG TAACAGCTTC TCGTACCAAT AGCTACGGTA CACCACGTCC AAATGCTGAA 3300

ATCTACAATA GCCTCTACGC AGCTAAAACA CGTACCTTTG GAAATGACTT CCAAGGCAAG 3360

TACGGTGGTG CCTTCCTTGA TGAATTGAAG GCAAAATACC CAGCAATCTT TGAGCGCGTG 3420

CAGATTTCAA ACGGCCGTAA ATTGACTACC AATGAGAAAA TCACGCAATG GTCAGCCAAG 3480

TATTTCAATG GAAGCAATAT CCAAGGTACT GGAGCTCGCT ATGTCCTACA AGATAACGCT 3540

ACCAACCAAT ACTTCAGCGT CAAAGCAGGT CAAACCTTCC TTCCTAAACA AATGACTGAA 3600

ATTACTGGAA GTGGTTTCCG TAGGGTTGGA GATGATGTCC AATACCTCTC AATTGGTGGC 3660

TACCTTGCTA AGAATACCTT TATTCAAGTC GGTGCCAACC AGTGGTATTA CTTTGATAAG 3720

AATGGCAACA TGGTCACAGG TGAGCAGGTC ATTGATGGCA AGAAATACTT CTTCCTAGAC 3780

AATGGTCTCC AGCTACGTCA TGTCCTTCGC CAAGGTAGTG ATGGTCATGT GTATTATTAC 3840

GATCCTAAAG GGGTTCAGGC CTTTAACGGA TTTTATGATT TTGCGGGTCC TCGCCAAGAC 3900

GTTCGCTACT TTGATGGCAA CGGTCAAATG TATCGTGGCC TCCACGACAT GTATGGCACA 3960

ACCTTCTATT TTGATGAAAA GACTGGTATT CAAGCCAAAG ACAAGTTCAT CCGTTTTGCG 4020

GACGGACGCA CGCGTTACTT CATCCCAGAT ACAGGAAATC TCGCAGTCAA CCGATTTGCG 4080

CAAAATCCTG AGAACAAGGC TTGGTATTAC CTCGATAGCA ACGGTTATGC CGTGACAGGA 4140

CTACAAACCA TTAACGGTAA GCAGTATTAC TTTGACAATG AAGGACGTCA GGTTAAGGGA 4200

CACTTTGTCA CTATCAATAA CCAACGTTAC TTCCTTGATG GTGATAGTGG TGAAATTGCT 4260

CCGTCACGCT TTGTGACGGA AAACAACAAG TGGTACTATG TCGATGGCAA TGGTAAACTG 4320

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GTCAAGGGTG CCTGGGCCAA CGGCCGTTAC TATGATGGTG ACTCAGGTCA GGCCGTAAGC 4440

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GTAACAGGTC TTCAAAATAT TAACAATAAA GTTTACTATT TTGGTAGCAA TGGTGCTCAA 4560

GTCAAAGGTA AATTGCTCAC TGTCCAAGGT AAGAAATGTT ACTTTGATGC CCACACAGGT 4620

GAGCAAGTGG TAAACCGCTT TGTCGAAGCT GCACGTGGCT GCTGGTATTA CTTTAACTCA 4680

GCTGGCCAAG CAGTGACTGG ACAACAGGTC ATCAATGGTA AACAACTTTA CTTCGACGGT 4740

TCAGGTCGTC AAGTTAAAGG ACGTTATGTT TATGTTGGTG GTAAACGACT CTTCTGCGAT 4800

GCCAAAACTG GTGAATTGAG ACAGCGTCGC TAATTAATAT GTACTTTAAA AAT 4853

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1577 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Streptococcus salivarius

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Glu Asn Lys Val Arg Phe Lys Leu His Lys Val Lys Lys Asn Trp
1 5 10 15

Val Thr Ile Gly Val Thr Thr Leu Ser Met Val Ala Leu Ala Gly Gly
20 25 30

Ser Leu Leu Ala Gln Gly Lys Val Glu Ala Asp Glu Thr Ser Ala Pro
35 40 45

Asn Gly Asp Gly Leu Gln Gln Leu Ser Glu Asp Gly Thr Ala Ser Leu
50 55 60

Val Thr Thr Thr Thr Val Thr Glu Gln Ala Ser Ala Gln Ala Ser Val
65 70 75 80

Ser Ala Val Ala Thr Ala Ser Val Ser His Glu Thr Ser Phe Gln Ala
85 90 95

Ala Thr Ser Ala Val Ser Gln Glu Ala Thr Ala Gln Ala Gln Thr Ser
100 105 110

Pro Val Ala Ser Gln Glu Val Ala Val Ser Ser Gln Thr Gln Ser Ser
115 120 125

Gly Gln Glu Thr Gln Thr Thr Glu Gln Val Ser Gln Gly Gln Thr Ser
130 135 140

Thr Gln Val Ala Gly Gln Thr Ser Ala Gln Ser Thr Pro Ser Val Thr
145 150 155 160

Glu Gln Ala Arg Pro Arg Val Leu Thr Asn Ala Ala Pro Ala Ile Ala
165 170 175

Thr Arg Ala Ala Asp Ser Thr Ile Arg Ile Asn Ala Asn Arg Asn Thr
180 185 190

Asn Ile Thr Ile Thr Ala Ser Gly Thr Thr Pro Asn Val Thr Ile Ile
195 200 205

Thr Gly Pro Asn Thr Pro Lys Pro Asn Val Thr Val Thr Ser Pro Asn
210 215 220

Gly Thr Arg Pro Asn Val Thr Ile Val Thr Gln Pro Asn Gln Pro Asn
225 230 235 240

Lys Pro Val Gln Pro Ser Gln Pro Ser Gln Pro Asn Lys Pro Val Gln
245 250 255

Pro Asn Gln Pro Ser Leu Asp Tyr Lys Pro Val Ala Ser Asn Leu Lys
260 265 270

Thr Ile Asp Gly Lys Gln Tyr Tyr Val Glu Asn Gly Val Val Lys Lys
275 280 285

Asn Ala Ala Ile Glu Leu Asp Gly Arg Leu Tyr Tyr Phe Asp Glu Thr
290 295 300

Gly Ala Met Val Asp Gln Ser Lys Pro Leu Tyr Arg Ala Asp Ala Ile
305 310 315 320

Pro Asn Asn Ser Ile Tyr Ala Val Tyr Asn Gln Ala Tyr Asp Thr Ser
325 330 335

Ser Lys Ser Phe Glu His Leu Asp Asn Phe Leu Thr Ala Asp Ser Trp
340 345 350

Tyr Arg Pro Lys Gln Ile Leu Lys Asp Gly Lys Asn Trp Thr Ala Ser
355 360 365

Thr Glu Lys Asp Tyr Arg Pro Leu Leu Met Thr Trp Trp Pro Asp Lys
370 375 380

Val Thr Gln Val Asn Tyr Leu Asn Tyr Met Ser Gln Gln Gly Phe Gly
385 390 395 400

Asn Lys Thr Tyr Thr Thr Asp Met Met Ser Tyr Asp Leu Ala Ala Ala
405 410 415

Ala Glu Thr Val Gln Arg Gly Ile Glu Glu Arg Ile Gly Arg Glu Gly
420 425 430

Asn Thr Thr Trp Leu Arg Gln Leu Met Ser Asp Phe Ile Lys Thr Gln
435 440 445

Pro Gly Trp Asn Ser Glu Ser Glu Asp Asn Leu Leu Val Gly Lys Asp
450 455 460

His Leu Gln Gly Gly Ala Leu Thr Phe Leu Asn Asn Ser Ala Thr Ser
465 470 475 480

His Ala Asn Ser Asp Phe Arg Leu Met Asn Arg Thr Pro Thr Asn Gln
485 490 495

Thr Gly Thr Arg Lys Tyr His Ile Asp Arg Ser Asn Gly Gly Tyr Glu
500 505 510

Leu Leu Leu Ala Asn Asp Ile Asp Asn Ser Asn Pro Ala Val Gln Ala
515 520 525

Glu Gln Leu Asn Trp Leu His Tyr Ile Met Asn Ile Gly Ser Ile Leu
530 535 540

Gly Asn Asp Pro Ser Ala Asn Phe Asp Gly Val Arg Ile Asp Ala Val
545 550 555 560

Asp Asn Val Asp Ala Asp Leu Leu Gln Ile Ala Ser Asp Tyr Phe Lys
565 570 575

Glu Lys Tyr Arg Val Ala Asp Asn Glu Ala Asn Ala Ile Ala His Leu
580 585 590

Ser Ile Leu Glu Ala Trp Ser Tyr Asn Asp His Gln Tyr Asn Lys Asp
595 600 605

Thr Lys Gly Ala Gln Leu Ser Ile Asp Asn Pro Leu Arg Glu Thr Leu
610 615 620

Leu Thr Thr Phe Leu Arg Lys Ser Asn Tyr Arg Gly Ser Leu Glu Arg
625 630 635 640

Val Il Thr Asn Ser Leu Asn Asn Arg Ser Ser Glu Gln Lys His Thr
645 650 655

Pro Arg Asp Ala Asn Tyr Ile Phe Val Arg Ala His Asp Ser Glu Val
660 665 670

Gln Ala Val Leu Ala Asn Ile Ile Ser Lys Gln Ile Asn Pro Lys Thr
675 680 685

Asp Gly Phe Thr Phe Thr Met Asp Glu Leu Lys Gln Ala Phe Glu Ile
690 695 700

Tyr Asn Ala Asp Ile Ala Lys Ala Asp Lys Lys Tyr Thr Gln Tyr Asn
705 710 715 720

Ile Pro Ala Ala Tyr Ala Thr Met Leu Thr Asn Lys Asp Ser Ile Thr
725 730 735

Arg Val Tyr Tyr Gly Asp Leu Phe Thr Asp Asp Gly Gln Tyr Met Ala
740 745 750

Glu Lys Ser Pro Tyr Tyr Asn Ala Ile Asp Ala Leu Leu Arg Ala Arg
755 760 765

Ile Lys Tyr Val Ala Gly Gly Gln Asp Met Lys Val Thr Lys Leu Asn
770 775 780

Gly Tyr Glu Ile Met Ser Ser Val Arg Tyr Gly Lys Gly Ala Glu Glu
785 790 795 800

Ala Asn Gln Leu Gly Thr Ala Glu Thr Arg Asn Gln Gly Met Leu Val
805 810 815

Leu Thr Ala Asn Arg Pro Asp Met Lys Leu Gly Ala Asn Asp Arg Leu
820 825 830

Val Val Asn Met Gly Ala Ala His Lys Asn Gln Ala Tyr Arg Pro Leu
835 840 845

Leu Leu Ser Lys Ser Thr Gly Leu Ala Thr Tyr Leu Lys Asp Ser Asp
850 855 860

Val Pro Ala Gly Leu Val Arg Tyr Thr Asp Asn Gln Gly Asn Leu Thr
865 870 875 880

Phe Thr Ala Asp Asp Ile Ala Gly His Ser Thr Val Glu Val Ser Gly
885 890 895

Tyr Leu Ala Val Trp Val Pro Val Gly Ala Ser Glu Asn Gln Asp Ala
900 905 910

Arg Thr Lys Ala Ser Ser Thr Lys Lys Gly Glu Gln Val Phe Glu Ser
915 920 925

Ser Ala Ala Leu Asp Ser Gln Val Ile Tyr Glu Gly Rhe Ser Asn Phe
930 935 940

Gln Asp Phe Val Lys Thr Pro Ser Gln Tyr Thr Asn Arg Val Ile Ala
945 950 955 960

Gln Asn Ala Lys Leu Phe Lys Glu Trp Gly Ile Thr Ser Phe Glu Phe
965 970 975

Ala Pro Gln Tyr Val Ser Ser Gln Asp Gly Thr Phe Leu Asp Ser Ile
980 985 990

Ile Glu Asn Gly Tyr Ala Phe Glu Asp Arg Tyr Asp Ile Ala Met Ser
995 1000 1005

Lys Asn Asn Lys Tyr Gly Ser Leu Lys Asp Leu Met Asp Ala Leu Arg
1010 1015 1020

Ala Leu His Ala Glu Gly Ile Ser Ala Ile Ala Asp Trp Val Pro Asp
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Gln Ile Tyr Asn Leu Pro Gly Lys Glu Val Val Thr Ala Ser Arg Thr
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Asn Ser Tyr Gly Thr Pro Arg Pro Asn Ala Glu Ile Tyr Asn Ser Leu
1060 1065 1070

Tyr Ala Ala Lys Thr Arg Thr Phe Gly Asn Asp Phe Gln Gly Lys Tyr
1075 1080 1085

Gly Gly Ala Phe Leu Asp Glu Leu Lys Ala Lys Tyr Pro Ala Ile Phe
1090 1095 1100

Glu Arg Val Gln Ile Ser Asn Gly Arg Lys Leu Thr Thr Asn Glu Lys
1105 1110 1115 1120

Ile Thr Gln Trp Ser Ala Lys Tyr Phe Asn Gly Ser Asn Ile Gln Gly
1125 1130 1135

Thr Gly Ala Arg Tyr Val Leu Gln Asp Asn Ala Thr Asn Gln Tyr Phe
1140 1145 1150

Ser Val Lys Ala Gly Gln Thr Phe Leu Pro Lys Gln Met Thr Glu Ile
1155 1160 1165

Thr Gly Ser Gly Phe Arg Arg Val Gly Asp Asp Val Gln Tyr Leu Ser
1170 1175 1180

Ile Gly Gly Tyr Leu Ala Lys Asn Thr Phe Ile Gln Val Gly Ala Asn
1185 1190 1195 1200

Gln Trp Tyr Tyr Phe Asp Lys Asn Gly Asn Met Val Thr Gly Glu Gln
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Val Ile Asp Gly Lys Lys Tyr Phe Phe Leu Asp Asn Gly Leu Gln Leu
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Arg His Val Leu Arg Gln Gly Ser Asp Gly His Val Tyr Tyr Tyr Asp
1235 1240 1245

Pro Lys Gly Val Gln Ala Phe Asn Gly Phe Tyr Asp Phe Ala Gly Pro
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Arg Gln Asp Val Arg Tyr Phe Asp Gly Asn Gly Gln Met Tyr Arg Gly
1265 1270 1275 1280

Leu His Asp Met Tyr Gly Thr Thr Phe Tyr Phe Asp Glu Lys Thr Gly
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Ile Gln Ala Lys Asp Lys Phe Ile Arg Phe Ala Asp Gly Arg Thr Arg
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Tyr Phe Ile Pro Asp Thr Gly Asn Leu Ala Val Asn Arg Phe Ala Gln
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Asn Pro Glu Asn Lys Ala Trp Tyr Tyr Leu Asp Ser Asn Gly Tyr Ala
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Val Thr Gly Leu Gln Thr Ile Asn Gly Lys Gln Tyr Tyr Phe Asp Asn
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Glu Gly Arg Gln Val Lys Gly His Phe Val Thr Ile Asn Asn Gln Arg
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Tyr Phe Leu Asp Gly Asp Ser Gly Glu Ile Ala Pro Ser Arg Phe Val
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Thr Glu Asn Asn Lys Trp Tyr Tyr Val Asp Gly Asn Gly Lys Leu Val
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Lys Gly Ala Gln Val Ile Asn Gly Asn His Tyr Tyr Phe Asn Asn Asp
1410 1415 1420

Tyr Ser Gln Val Lys Gly Ala Trp Ala Asn Gly Arg Tyr Tyr Asp Gly
1425 1430 1435 1440

Asp Ser Gly Gln Ala Val Ser Asn Gln Phe Ile Gln Ile Ala Ala Asn
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Gln Trp Ala Tyr Leu Asn Gln Asp Gly His Lys Val Thr Gly Leu Gln
1460 1465 1470

Asn Ile Asn Asn Lys Val Tyr Tyr Phe Gly Ser Asn Gly Ala Gln Val
1475 1480 1485

Lys Gly Lys Leu Leu Thr Val Gln Gly Lys Lys Cys Tyr Phe Asp Ala
1490 1495 1500

His Thr Gly Glu Gln Val Val Asn Arg Phe Val Glu Ala Ala Arg Gly
1505 1510 1515 1520

Cys Trp Tyr Tyr Phe Asn Ser Ala Gly Gln Ala Val Thr Gly Gln Gln
1525 1530 1535

Val Ile Asn Gly Lys Gln Leu Tyr Phe Asp Gly Ser Gly Arg Gln Val
1540 1545 1550

Lys Gly Arg Tyr Val Tyr Val Gly Gly Lys Arg Leu Phe Cys Asp Ala
1555 1560 1565

Lys Thr Gly Glu Leu Arg Gln Arg Arg
1570 1575

LIST OF REFERENCES

1. Radojevic et al. 1994 Aust J Agric Res 45, 901-12.

2. Jacques NA, Giffard PM, "The Glycosyltransferases of Oral Streptococci" Todays Life Science 1991; 3: 40-6.

3. Walker GJ, Jacques NA, "Polysaccharides of Oral Streptococci" In: Reizer J, Peterkofsky A, Eds. "Sugar Transport and Metabolism in Gram-Positive Bacteria". Chichester: Ellis Horwood, 1987; 39-68.

4. Gilmore KS, Russell RRB, Ferretti JJ, "Anaylsis of the Streptococcus downei gtfS gene, which specifies a glucosyltransferase that synthesises soluble glucans". Infect Immun 1990; 58: 2452-8.

5. Giffard PM, Simpson CL, Milward CP, Jacques NA, "Molecular characterization of a cluster of at least two glucosyltransferase genes in *Streptococcus salivarius* ATCC25975". J. Gen. Microbiol. 1991; 137:2577-93.

6. Giffard PM, Allen DM, Milward CP, Simpson CL, Jacques NA, "Sequence of the GtfK of *Streptococcus salivarius* ATCC25975 and the evolution of the gtf genes of oral streptococci". J Gen Microbiol 1993; 139:1511-22.

7. Pitty LS, Giffard PM, Gilpin ML, Russell RRB and Jacques NA, 1989. "Cloning and expression of glycosyltransferase C gene (gtfC) from *Streptococcus mutans* LM7. Infect Immun 55: 2176-2182.

8. Silliary TS, Berman ML, and Enquist LW, 1984. "Experiments with Gene fusions". Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

9. Jacques N.A. 1983. Membrane perturbation by cerulenin modulates glucosyltransferase secretion and acetate uptake by *Streptococcus salivarius*. J. Gen. Micriobial. 129 : 3293-3302.

10. Maniatis T, Fritsch EF, and Sambrook J. (1989). "Molecular Cloning; a laboratory manual. Second edition. Cold Spring Harbor Laboratory Press, N.Y.

THE CLAIMS

1. A plant containing bacterial DNA which codes for a glucosyltransferase which catalyses the formation of glucans from sucrose.

2. A plant according to claim 1 wherein the bacterial DNA is primer independent.

3. A plant according to claim 1 where the glucosyltransferase catalyses the formation of soluble glucans.

4. A DNA comprising a sequence according to SEQ ID NO: 1.

5. A DNA having a sequence which is a variant of SEQ ID. NO: 1.

6. A protein comprising an amino acid sequence according to SEQ ID NO: 2.

7. A protein comprising an amino-acid sequence which is a variant of SEQ ID NO:2.

8. The plasmid pGSG501 containing λC-13 DNA.

9. The plasmid pGSG502 containing λC-13 DNA.

10. A plant containing DNA comprising a sequence according to SEQ ID NO: 1.

11. A plant containing DNA having a sequence which is a variant of DNA SEQ ID NO: 1.

12. A plant expressing a protein comprising an amino acid sequence according to SEQ ID NO: 2.

13. A plant expressing a protein comprising an amino acid sequence which is a variant of SEQ ID NO: 2.

AMENDED CLAIMS

[received by the International Bureau on 18 January 1996 (18.01.96); original claims 2,3 cancelled; original claims 1,5,7,11,13 amended and renumbered; new claims 12-14 added; claim 4 renumbered as claim 2 claim 6 renumbered as claim 4 claims 8-10 renumbered as claims 6-8 claim 12 renumbered as claim 10 (2 pages)]

1. A plant containing primer independent bacterial DNA which codes for a glucosyltransferase which catalyses the formation of soluble glucans from sucrose.

2. A DNA comprising a sequence according to SEQ ID NO: 1.

3. A DNA having a sequence which is a variant of SEQ ID. NO: 1, in which minor alterations have been made compared to SEQ ID. NO: 1 resulting in a sequence which is not identical to SEQ ID. NO: 1.

4. A protein comprising an amino acid sequence according to SEQ ID NO: 2.

5. A protein comprising an amino-acid sequence which is a variant of SEQ ID NO: 2, in which minor alterations have been made compared to SEQ ID NO: 2 resulting in a sequence which is not identical to SEQ ID NO. 2.

6. The plasmid pGSG501 containing λC-13 DNA.

7. The plasmid pGSG502 containing λC-13 DNA.

8. A plant containing DNA comprising a sequence according to SEQ ID NO: 1.

9. A plant containing DNA according to claim 3.

10. A plant expressing a protein comprising an amino acid sequence according to SEQ ID NO: 2.

11. A plant expressing a protein comprising an amino acid sequence according to claim 5.

12. A method of improving the level of stored carbohydrate in a pasture plant with low levels, comprising inserting a DNA according to claim 2 or 3 into the plant so that the plant expresses a protein according to claim 4 or 5 in active form.

13. A method of preventing degradation of stored carbohydrate during plant senescence comprising inserting a DNA according to claim 2 or 3 into the plant so that the plant expresses a protein according to claim 4 or 5 in active form.

14. Dextran, when produced from a plant according to claim 1, or any one of claims 8 to 11.

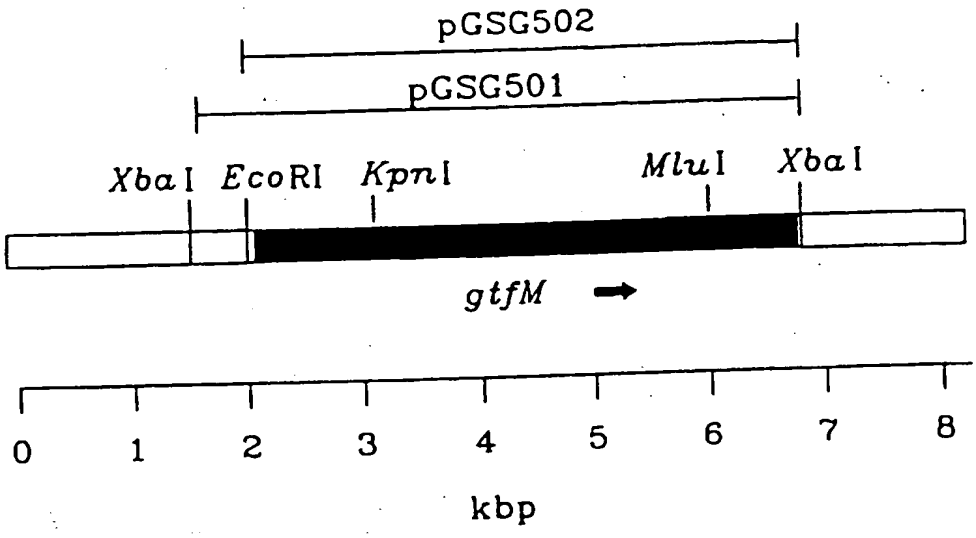
pGSG502
pGSG501
XbaI
EcoRI
KpnI
MluI
XbaI
gtfM
0
1
2
3
4
5
6
7
8
kbp

Figure 1

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 95/00527

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N 15/54, 9/10; A01H 5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IC6: C12N (keywords below); Chemical Abstracts (keywords below)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
BIOT (keywords below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
cas on-line: keywords: (EC-2.4.1.5 or EC 2.4.1.125 or glucosyl()trans: or GTF or glucosyltrans: or transgl:) (SS1) and 3/SC and 1989-1995 or (sucrose:or plant:)
WPAT: keywords: glycosyl()trans: or GTF or glucosyltrans: or transglucosylase: and C12N/IC and (sucrose or plant)
continued

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Infection and Immunity (Feb 1995) vol. 63, no. 2, pages 609-621, C.L. Simpson et al: "Streptococcus salivarius ATCC 25975 possesses at least two genes coding for primer-independent glucosyltransferases". (See whole document, in particular Abstract, figures 2(b) and 3)	4-9
X	WO, A, 90/02484 (Washington University) 22 March 1990 (22.03.90) (see in particular pages 25-26, examples 4, 5, 11 and claim 18)	1
X	WO, A, 89/12386 (Calgene Inc) 28 December 1989 (28.12.89) (see Abstract, page 8, line 33-page 9, line 1)	1

☐ Further documents are listed in the continuation of Box C

☒ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 December 1995	4 DECEMBER 1995
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929	Authorized officer **KAREN AYERS** Telephone No.: (06) 283 2082

Form PCT/ISA/210 (second sheet) (July 1992) copteb

INTERNATIONAL SEARCH [redacted]RT

[redacted]ternational Application No.
PCT/AU 95/00527

C (Continuati n) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	J. Gen. Microbiology (1993) vol.139, pages 1511-1522, P.M. Giffard et al: "Sequence of the gtfK gene of Streptococcus salivarius ATCC 25975 and evolution of the gtf genes of oral streptococci". (See whole document).	5,7 ----- 1-3
Y	Aust. J. Agric. Res., (1994) volume 45, pages 901-12, I. Radojevic et al: "Chemical composition and in vitro digestibility of lines of Lolium perenne selected for high concentrations of water-soluble carbohydrate" (See, in particular, page 910 third full paragraph)	1-3
Y	WO, A, 94/11520 (Zeneca Ltd) 26 May 1994 (26.05.94) (see page 2, line 25-page 3, line 10)	1-3

INTERNATIONAL SEAR[redacted]REPORT

[redacted]

...ternational Application No.
PCT/AU 95/00527

Box I **Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Box B (continuation)

BIOT: keywords: SSI (see above) and (A1/CL or E5/CL) and (sucrose # or plant)

STN search: (a) TGGCACAAGACCAAA

(b) TTACTAAGCTTAA

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.
PCT/AU 95/00527

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
WO	89/12386	AU	38520/89	IL	90713
WO	94/11520	AU	54285/94	GB	9223454

END OF ANNEX